

-- Some amino acid substitutions are preferably "conservative", with residues replaced with physicochemically similar residues, such as Gly/Ala, Asp/Glu, Val/Ile/Leu, Lys/Arg, Asn/Gln and Phe/Trp/Tyr. Analogs having such conservative substitutions typically retain substantial the SP168 receptor binding activity. Other analogs, which have non-conservative substitutions such as Asn/Glu, Val/Tyr and His/Glu, may substantially lack such activity.

A² Nevertheless, such analogs are useful because they can be used as antigens to elicit production of antibodies in an immunologically competent host. Because these analogs retain many of the epitopes (antigenic determinants) of the wild-type receptors from which they are derived, many antibodies produced against them can also bind to the active-conformation or denatured wild-type receptors.

Accordingly, such antibodies can also be used, e.g., for the immunopurification or immunoassay of the wild-type receptors. --

(3) Please replace the paragraph on page 25, line 15 with the following rewritten paragraph:

A³ -- The SP168 receptor cDNA was subcloned into the pcDNA3.1 expression vector (Invitrogen) and transiently co-transfected with Gq/i3 into CHO-DHFR (ATCC CRL-9096) cells using lipofectamine (Gibco- BRL). pCDNA 3.1 vector DNA was also co-transfected with Gq/i3 as a negative control. A variety of nucleotides, RBI small moleculars, and in-house peptide collection were screened. Twelve compounds were identified to be the agonists of the SP168 receptor. Their fluorecence change with different amount of the compounds were detected, the data were processed using Prism software to obtain their EC50 value. Other agonists can be identified using similar approaches. To identify antagonists of the SP168 receptor, two-addition approach was adapted. First, the compounds to be screened were added to the SP168 receptor transfected cells, after 5 minutes 100 nM of ADP were added as second addition and the fluorescence changes were recorded. In this assay antagonists inhibit the fluorecence signal of ADP. Using this method, reactive blue 2, suramin and 2', 5'-ADP were found to be antagonists of the SP168 receptor with IC50 of 4.78 μ M, 13.2 μ M and 36.9 μ M. --

(4) Please replace the paragraph on page 25, line 30 through page 26, line 4 with the following rewritten paragraph:

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A - - The SP168 receptor cDNA was stably transfected into CHO-DHFR cells according to standard procedures. The SP168 receptor stable cell line and wild type cells grown on 12-well plates were incubated for 2 hr with 200 μ l of medium plus 5 μ Ci of [3 H]adenine/ml. Then 50 μ l of Hepes (250 mM, pH 7.5, 50 μ M Forskolin, 200 μ M IBMX) with the compound to be screened was added to the cells and incubated for 10 minutes at 37 °C. Incubations were terminated by addition of 0.8 ml of 5% trichloroacetic acid. [3 H]cAMP was purified using Dowex and alumina chromatography as previously described (Harden et al., Mol. Pharmacol. 21:570-580, 1982). - -

(5) Please replace the paragraph on page 26, line 12 with the following rewritten paragraph:

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A - - Cells were detached from the tissue culture plates by the addition of 5 ml cell dissociation solution (Sigma C5914) per plate. Cells from 10 plates were combined and centrifuged at 1000 x g for 5 minutes. The resulting cell pellet was resuspended in 25 ml of 5 mM HEPES (pH 7.4) containing 1X Complete Protease Inhibitor Cocktail (Boehringer Mannheim) and incubated at room temperature for 15 minutes. The cell suspension was then centrifuged for 15 minutes at 4°C at 11000xg using a Sorvall SS34 rotor. The resulting cell membrane pellet was resuspended in 5 mM HEPES containing 1X Protease Inhibitor Cocktail. The cell suspension was drawn five times through a syringe equipped with a 23 gauge needle, to ensure uniformity of membranes, and frozen in 200 μ l aliquots in liquid nitrogen and stored at -80°C. - -

(6) Please replace the paragraph on page 27, line 31 through page 28, line 13 with the following rewritten paragraph:

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-- NIH3T3 cells stably expressing the SP168 receptor were cultured to confluence in 6 or 12 well plates. The cells were then equilibrated for 16-24 hours in 1-2 μ Ci/ml myo[3 H]-inositol (NEN, cat# NET 114A) in complete culture media. The plates were then washed with warm (37°C) PBS. Cells were stimulated for 45-60 min at 37 °C with agonist (.01-1000 nM ADP) in the presence or absence of sample compound to be tested for antagonism. When screening for agonists, cells were stimulated with the test compounds alone. The test compounds were dissolved in PBS containing 20 mM LiCl and 1 mM CaCl₂. Reaction was stopped by aspirating the PBS and the cells lysed by adding 1 ml 0.4 M perchloric acid to each well and incubating the plates for 10-15 under refrigeration. 1.0 ml of lysates from wells were added to 0.5 ml of neutralizing solution (0.72 M KOH, 0.6 M KHCO₃) in 12x75 mm tubes. The tubes were shaken and then centrifuged for 5 minutes at 3000 x g. Dowex columns of 1 ml of a 50:50 slurry of Dowex in water (Formate form, BioRad AG1-x8 resin, 100-200 mesh cat# AG 140-1444) were prepared and 1 ml samples of the supernatants and 3 ml of water were added to each column. The columns were washed 2x with 10 ml water and then inositol phosphates were eluted from the columns directly into scintillation vials with 4 ml 1.0 M Ammonium formate, 0.1 M formic acid. After the addition of 10 ml scintillation fluid the samples were subjected to liquid scintillation spectroscopy (Beckman Scintillation Counter LS3801). --

(7) Please replace the paragraph on page 28, line 31 through page 29, line 4 with the following rewritten paragraph:

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-- The tissues used in this study were removed at surgery or at autopsy, fixed in 10% neutral buffered formalin for 16-24 hours, processed and embedded in paraffin. Serial 4 micron sections were then stained with hematoxylin and eosin or used in the in situ hybridization studies. The samples were evaluated for diagnostic verification and grading. Adjacent serial sections were then screened for the presence of preserved RNA by hybridization with an antisense beta-actin control riboprobe. Only tissues that passed the hybridization test with similar levels of

A7 signal were used in subsequent hybridization analyses. After hybridization, the slides were evaluated for interpretation of the *in situ* hybridization signal. - -

(8) Please replace the paragraph on page 29, line 5 with the following rewritten paragraph:

A8 - - *In situ* hybridization was carried out using [³³P]-UTP labeled cRNA probes. These probes were transcribed using PCR-generated fragments with sense and antisense transcription initiation sites (T3/T7) included on the PCR primers. The labeled probes were subjected to PAGE purification to determine size and purity. Paraffin-embedded tissue sections were digested with proteinase K and hybridized overnight at 60°C with 8 X 10⁸ dpm/ml of labeled probe. The sections were then treated with RNase A and washed for 2 hr in 0.1X SSC at 60°C. The slides were dipped in Kodak NTB emulsion and exposed for 2 weeks at 4°C. The slides were developed with Kodak D-19 developer, fixed in Kodak fixer, stained with hematoxylin and eosin, and coverslipped. - -

(9) Please replace the paragraph on page 29, line 19 with the following rewritten paragraph:

sub B1 A9 ~~-- The hybridization signals obtained for the SP 168 antisense cRNA probe were relatively consistent in all the normal tissues, although there appeared to be regional differences in the intensity of the signals. In all regions, astrocytes were the only brain cell type which exhibited appreciable hybridization signals. Labeled astrocytes were visible in both the gray and white matter, and accumulations of silver grains were also observed over perivascular astrocytes and astrocytes in subependymal regions. In terms of regional differences, the hybridization signals obtained with the antisense cRNA probe were most intense over astrocytes in temporal cortex, substantia nigra pars reticulata, and amygdala. Moderate signals were observed over thalamic astrocytes, while spinal cord and caudate nucleus contained only weak hybridization signals. In all tissues, only a subpopulation of astrocytes appeared to be labeled. - -~~

(10) Please replace the paragraph on page 29, line 30 with the following rewritten paragraph:

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-- The sections from the disease-related tissues tended to exhibit lower hybridization signals than those from normal tissues. Although the cell density appeared comparable, there were fewer labeled astrocytes in the temporal cortex of Alzheimer's samples. Those that did exhibit hybridization signal also contained fewer silver grains than did their normal counterparts. Similar observations were true for the amygdala from Alzheimer's samples, and for the substantia nigra pars reticulata of the Parkinson's samples. --